COELIAC DISEASE

Cross linking to tissue transglutaminase and collagen favours gliadin toxicity in coeliac disease

W Dieterich, B Esslinger, D Trapp, E Hahn, T Huff, W Seilmeier, H Wieser, D Schuppan

See end of article for authors' affiliations

Correspondence to: Dr W Dieterich, Department of Medicine I, University of Erlangen-Nuernberg, Glueckstrasse 10, Hofgebäude Labor Prof Schuppan, Erlangen 91054, Germany; walburga.dieterich@med1.imed.uni-erlangen.de

Revised version received 23 August 2005
Accepted for publication 20 September 2005
Published online first 27 September 2005

Gut 2006;**55**:478–484. doi: 10.1136/gut.2005.069385

Background and aims: Intestinal inflammation in coeliac disease is driven by the gluten fraction of wheat proteins. Deamidation or cross linking of gluten peptides by tissue transglutaminase (tTG), the coeliac disease autoantigen, creates potent T cell stimulatory peptides. Therefore, our aim was to identify the reaction patterns of gluten peptides, intestinal extracellular matrix proteins, and tTG.
Methods: tTG activity was analysed by incorporation of monodansyl cadaverine into gliadins. Fluorescence labelled tTG reactive short gliadin peptides were used to demonstrate their deamidation and explore their cross linking patterns with tTG itself or extracellular matrix proteins. Patient sera and controls were checked for autoantibodies to matrix proteins.
Results: Gliadins α1–α11, γ1–γ6, ω1–ω3, and ω5 were substrates for tTG. tTG catalysed the cross linking of gliadin peptides with interstitial collagen types I, III, and VI. Coeliac patients showed increased antibody titres against the collagens I, III, V, and VI.
Conclusions: tTG formed high molecular weight complexes with all tested gliadins. As all tested gliadins were substrates for tTG, the tTG catalysed modifications were not restricted to single gliadin types and epitopes. Furthermore, haptenisation and long term immobilisation of gliadin peptides by tTG catalysed binding to abundant extracellular matrix proteins could be instrumental in the perpetuation of intestinal inflammation and some associated autoimmune diseases in coeliac disease.

Several years ago gliadins were identified as the aetiological agent of coeliac disease.[1 2] Gliadins are the alcohol soluble fraction of gluten, the storage proteins of wheat, which are characterised by a high content of glutamine and proline residues. Gliadins have been classified into the major fractions α, γ, and ω gliadins, and subdivided into their subcomponents α1–α11 γ1–γ6, ω1–3, and ω5.[3 4] Gliadin toxicity was confirmed in several studies, with the main focus on the amino terminal region of A-gliadin, a major component of α-gliadin.[5–9] Later, short peptides from α- and γ-gliadins, which stimulate intestinal T cells from coeliac patients, were identified. The peptides bind to human leucocyte antigen (HLA)-DQ2 or -DQ8, a necessary precondition for the development of coeliac disease. Binding of these peptides to HLA-DQ2 or -DQ8 and the resultant T cell stimulation is potentiated when distinct glutamine residues are deamidated by tissue transglutaminase (tTG),[10–17] the autoantigen in coeliac disease.[18] tTG is an ubiquitous cellular enzyme that is upregulated in wound healing, angiogenesis, and apoptosis where its main function is cross linking of proteins via creation of stable isopeptide bonds between a donor glutamine residue and an acceptor lysine residue.[19] However, a low pH favours tTG catalysed deamidation of donor glutamines instead of its incorporation into an isopeptid bond. Of particular interest is the finding that the glutamine-rich gliadins are excellent donor substrates for the otherwise highly substrate specific tTG.[20] The amino acid composition around glutamine residues was shown to be critical for the reactivity of tTG with the gliadin peptides as their positional change or substitution in these peptides can enhance or abolish HLA binding and T cell reactivity.[13 14 17 21] Evidence for the involvement of all gliadins in the pathogenesis of coeliac disease has been derived from the presence of antibodies against α-, γ-[22 23] and ω-gliadins,[24] and from the damaging potential of ω-gliadins.[25 26]

Recent reports described immune response to glutenins, which differ in structure from gliadins and which are insoluble in aqueous alcohol.[27–29] The heterogeneity in the T cell stimulatory properties of various gliadin or glutenin peptides in childhood and adult patients gave rise to the hypothesis that the early immune response in coeliacs is directed towards several gliadin and glutenin peptides, while longstanding inflammation favours a few immunodominant gliadin peptides, preferentially deamidated by tTG and thus binding more tightly to HLA-DQ2 and -DQ8.[28–30]

To date, the catalytic activity of tTG for the different gliadins has not been compared. Furthermore, while tTG mediated deamidation of distinct peptides from α- and γ-gliadins was described in detail,[10–17] no information on the nature of complexes between gliadins and tTG, and between gliadins and extracellular matrix components in the small intestine is available. Here we demonstrate that all investigated gliadins are good substrates for tTG. In addition, gliadin peptides can react with tTG, resulting in gliadin-tTG cross link and complex formation. Furthermore, tTG catalyses the binding of gliadin peptides to interstitial collagen types I, III, and VI, which suggests the generation of complex neoepitopes and long term immobilisation of pathogenic gliadins in the intestinal extracellular matrix. Haptenisation of gliadins to collagen is associated with increased titres of IgA antibodies to collagens in the sera of coeliac patients and may in part explain the occurrence of autoimmune phenomena in coeliac disease.

Abbreviations: ECM, extracellular matrix; ELISA, enzyme linked immunosorbent assay; HLA, human leucocyte antigen; RP-HPLC, reversed phase-high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, Tris buffered saline; TRITC, tetramethyl-rhodamine; tTG, tissue transglutaminase

METHODS

Isolation of gliadins

Gliadins were fractionated as described previously.[4] In brief, crude gliadin from German wheat cultivar ''Rektor'' was subjected to reversed phase-high performance liquid chromatography (RP-HPLC), using a Nucleosil C8 (5 µm, 30 mm) column. A linear gradient (flow rate 1 ml/min) was run from 16% to 42% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Single fractions of α1–α11, γ1–γ6, and ω1–ω3 and ω5 gliadin were collected and freeze dried. Assignment of isolated fractions to the different gliadin types by means of amino terminal sequencing has been described previously.[4 31 32]

Peptide synthesis

The following peptides derived from α2-gliadin[10] were synthesised on a peptide synthesiser (431A Applied Biosystems, Weiterstadt, Germany) by Fmoc chemistry and purified by two steps of preparative RP-HPLC[33]: α2(58–88): LQP FPQ PQL PYP QPQ LPY PQP QLP YPQ PQP F; α2(58–88, E65, E72, E79): LQP FPQ P$\underline{E}^{65}$L PYP QP$\underline{E}^{72}$ LPY PQP $\underline{E}^{79}$L PYP QPQ PF; α2(56–68): LQL QPF PQP QLP Y; α2(56–68, E59): LQL $\underline{E}^{59}$P FPQ PQL PY; α2(56–68, E63): LQL QPF P$\underline{E}^{63}$P QLP Y; α2(56–68, E65): LQL QPF PQP $\underline{E}^{65}$LP Y, with $\underline{E}$ denoting glutamic acid residues, corresponding to deamidated glutamines in the parent peptides. All peptides were chromatographically pure (>99%) and their masses confirmed by mass spectrometry.

Fluorometric assay for tTG catalysed incorporation of monodansyl cadaverine into gliadins

Incorporation of monodansyl cadaverine (N-(5-aminopentyl)-5-dimethylamino-1-naphthalinsulfonamide) (Sigma, Taufkirchen, Germany) into proteins results in enhanced fluorescence of the dansyl group, as described previously.[34] This phenomenon was used to determine substrate specificity of tTG for the isolated gliadin fractions, which is proportional to incorporation of monodansyl cadaverine (glutamine acceptor) into gliadins (glutamine donor). The corresponding gliadin fraction or gliadin peptide (20 µg) was incubated with 30 µM monodansyl cadaverine in a total volume of 100 µl 0.1 M Tris HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.5. Addition of 0.5 µg human recombinant tTG, isolated and characterised as described previously,[35] started the reaction which was followed for two minutes at 37°C. Excitation was set to 360 nm and the increase in fluorescence intensity measured at 550 nm with a fluorescence spectrophotometer (Photon Technology International, Canada). Crude gliadin (Sigma) was used as a positive control.

Fluorescence labelling of gliadin peptides

The amino terminal labelling of peptides with the fluorescent dye Alexa Fluor 488-carboxylic acid-succinimidyl ester (MoBiTec, Göttingen, Germany) was performed according to the manufacturer's recommendations. Briefly, 1 mg of the Alexa labelled amine reactive compound was dissolved in 100 µl dimethylsulfoxide at 10 mg/ml and incubated with the gliadin peptides in 0.1 M sodium bicarbonate buffer at equimolar ratios at room temperature for one hour. Separation of labelled gliadin peptides was carried out using a linear gradient from 0.1% (v/v) trifluoroacetic acid to 60% acetonitrile/0.1% trifluoroacetic acid within 90 minutes at a flow rate of 0.75 ml/min using a Beckman ODS Ultrasphere column (5 µm, 4.6×250 mm). Alexa labelled fractions showing fluorescence at 520 nm were collected and the identity of the peptides confirmed by amino acid analysis after acid hydrolysis and precolumn derivatisation with o-phthaldialdehyde/3-mercaptopropionic acid.

pH dependency of deamidation and cross linking of gliadin peptides

Alexa labelled gliadin peptides (0.2 µg) were incubated in 0.1 M Tris HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH varying from 6.0 to 7.5, with 0.5 µg of human recombinant tTG in a total volume of 15 µl for one hour at 37°C. The reaction was stopped by heating at 95°C for 10 minutes in denaturing gel loading buffer (1 M urea, 2% sodium dodecyl sulphate), and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Fluorescence detection of Alexa labelled gliadin peptides allowed determination of deamidated or cross linked peptides.

Incorporation of gliadin peptide α2(56–68) into extracellular matrix components in vitro and ex vivo

Human collagen types I, III, and VI were purified and characterised as described previously.[36–38] The collagens were dissolved at 1 mg/ml in 0.15 M acetic acid for collagen type I, in 0.01 M acetic acid for type V (Rockland, USA), or in 0.5 M acetic acid for types III and VI. Control experiments showed no interference of residual acetic acid with tTG activity. Fibronectin (Biotrend, Cologne, Germany) was dissolved in 50 mM Tris HCl, 150 mM sodium chloride, 5 mM $CaCl_2$, pH 7.5. The corresponding extracellular matrix (ECM) components (4 µg) were incubated with 0.2 µg of fluorescence (Alexa) labelled *α2(56–68) peptide in 50 mM Tris HCl, 150 mM sodium chloride, 5 mM $CaCl_2$, and pH was adjusted to 7.5. The reaction was started with 0.5 µg tTG in a total volume of 12 µl and allowed to proceed for 30 minutes at 37°C. After separation of reaction products by SDS-PAGE, the fluorescence of incorporated Alexa labelled peptides was visualised and protein bands detected after staining with Coomassie blue.

Cryosections of primate oesophagus (The Binding Site, Schwetzingen, Germany) were used for immunofluorescence staining. Oesophageal sections were used for most of the experiments as they demonstrated most clearly the subtle differences in the investigated staining patterns, while intestinal tissue yielded similar results of lower resolution. Affinity purified rabbit antibodies against collagen types I, III, and VI, fibronectin, and a monoclonal antibody against tTG (Quartett, Berlin, Germany) were diluted 1/50 or 1/20, respectively, in 0.1 M Tris HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.5 (Tris buffered saline (TBS)), and 0.1% bovine serum albumin, and incubated with the sections at room temperature in a humidified atmosphere for 30 minutes. The specimens were washed and further incubated with tetramethylrhodamine (TRITC) coupled swine antirabbit or rabbit antimouse antibody (DakoCytomation, Hamburg, Germany; dilution 1/50) in phosphate buffered saline at room temperature for 30 minutes. Immunofluorescence staining of matrix proteins or tTG was visualised in a Zeiss microscope equipped with a filter for TRITC. To demonstrate the binding of externally added tTG, sections were preincubated with 2 µg tTG prior to detection with the monoclonal anti-tTG antibody.

To investigate how far tTG reactive gliadin peptides bind to ECM components ex vivo, oesophageal sections were incubated with the Alexa labelled gliadin peptide *α2(56–68) alone (0.2 µg) or in combination with increasing amounts of human recombinant tTG (0.5–2.0 µg) in TBS and 0.1% bovine serum albumin, pH 7.4. Fluorescence of the Alexa labelled gliadin peptide was visualised with a 395 nm filter.

Enzyme linked immunosorbent assay (ELISA) for serum IgA antibodies to collagens

Ninety six well microtitre plates (Greiner, Frickenhausen, Germany) were coated with 1 µg/well of collagens I, III, V, and VI, or fibronectin in 100 µl of 50 mM Tris HCl, 150 mM NaCl, and 5 mM $CaCl_2$, pH 7.5, at 37°C for two hours. Wells were extensively washed with 50 mM Tris HCl, 150 mM NaCl, 10 mM ethylene-diaminetetraacetate, and 0.1% Tween-20, pH 7.4, and kept in washing buffer overnight at 4°C. Sera of patients with proven active coeliac disease, as determined by small intestinal biopsy and positive serum IgA autoantibodies to tTG (n = 16) or healthy controls (n = 15), were diluted 1/25 in 100 µl of the same buffer and incubated for one hour at room temperature. Afterwards the wells were washed and exposed to 100 µl peroxidase conjugated rabbit antihuman IgA (Dianova, Hamburg, Germany) diluted 1/4000 in the same buffer. After removal of unbound antibodies the colour was developed by addition of 100 µl of 0.1 M sodium citrate, 1 mg/ml o-phenylenediamine-hydrochloride (Sigma, Deisenhofen, Germany), and 0.007% H_2O_2 (Merck, Germany), pH 4.2, at room temperature for 30 minutes in the dark. Absorbances were read on an ELISA reader (MRX, Dynatech, Denkendorf, Germany) at 450 nm. All measurements were performed in duplicate.

Statistics

To compare titres of IgA autoantibodies from patients with coeliac disease with those of non-coeliac controls, a non-paired non-parametric Mann-Whitney U test was performed for each matrix protein. A p value of <0.05 was considered to be significant.

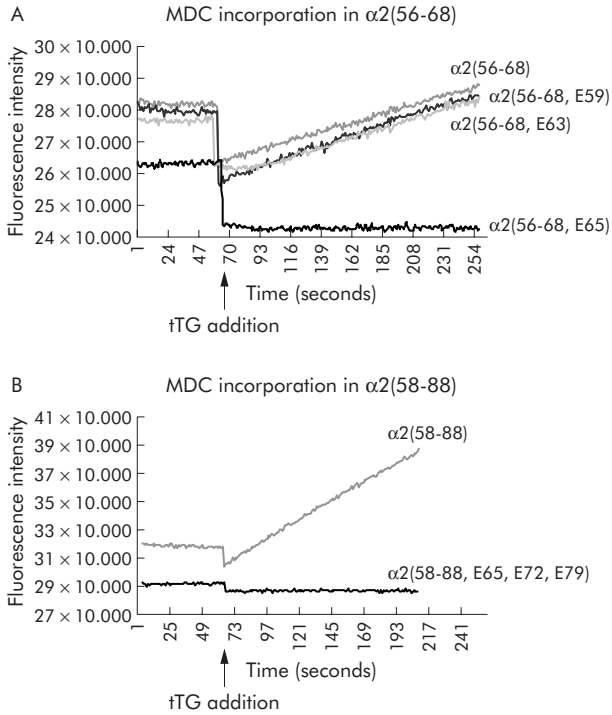

Figure 1 Site specific deamidation of gliadin peptides prevents tissue transglutaminase (tTG) catalysed incorporation of monodansyl cadaverine (MDC). Incorporation of MDC into gliadin peptides is dependent on specific glutamine residues in gliadins and detected by increased fluorescence intensity. (A) Only deamidation of the substrate residue Q65 in the gliadin peptide α2(56–68) resulted in failure of MDC incorporation. (B) Deamidation of the three tTG substrate residues Q65, Q72, and Q79 prevented tTG catalysed modification of the protease resistant gliadin peptide α2(56–88) which contains three of the immunodominant substrate sequences.

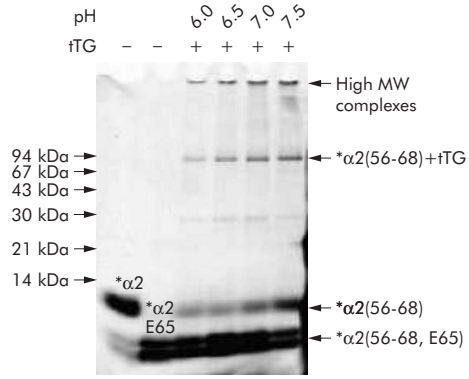

Figure 2 Effect of pH on cross linking of gliadin to tissue transglutaminase (tTG). The formation of cross links between tTG and fluorescence labelled peptide *α2(56–68) was investigated after separation by 18% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Increasing pH enhanced cross linking of peptide *α2(56–68) to tTG. The tTG monomer and oligo/multimers with the gliadin peptide attached are visualised as high molecular weight complexes. The two lanes on the left show the *α2(56–68) peptide and its deamidated variant which has a higher mobility than the non-deamidated parent peptide.

RESULTS

Confirmation of isolated gliadin fractions as substrates for tTG

Using a quantitative assay which is based on tTG catalysed incorporation of monodansyl cadaverine into gliadins followed by measurement of enhanced fluorescence intensity, all of the tested gliadins (α1–11, γ1–6, and ω1, 2, 3, and 5) were found to be good substrates for tTG, showing an increase in fluorescence reaching 50–100% of values obtained with crude gliadin as substrate (data not shown). No incorporation of monodansyl cadaverine was detected with the control substrates bovine serum albumin and lactalbumin.

Substrate specificity of gliadin peptides for tTG

The high selectivity of tTG for glutamine donor substrates is well known and the gliadin derived sequence PQPQLPY was described as high affinity substrate for tTG.[39] Using a fluorometric tTG activity assay, the absolute requirement of Q65 as reactive glutamine in the gliadin peptide α2(56–68; PQL QPF PQP QLP Y) was confirmed. This 13 amino acid peptide and its deamidated variants E59 and E63 were good substrates for tTG, while substitution of Q65 by E65 resulted in complete loss of reactivity with tTG, therefore confirming that modification of peptide α2(56–68) by tTG is strictly restricted to Q65 (fig 1A). Three copies of the preferred recognition motif are found in the protease resistant 31-mer α2(58–88; LQP FPQ PQL PYP QPQ LPY PQP QLP YPQ PQP F).[12] The multiple presence of this motif suggested that the sole deamidation of Q65 to E65 could not abolish reactivity of this larger peptide with tTG. Accordingly, only replacement of Q65, Q72, and Q79 by glutamic acid in the extended peptide α2(58–88) abolished reactivity with tTG (fig 1B), confirming the PQPQLPY motif as the sole tTG recognition sequence in these gliadin peptides.

pH dependency of tTG catalysed cross linking

As low pH values can prevail in inflamed intestinal mucosa,[13] tTG catalysed modification of α2(56–68) was checked with pH values adjusted to 6.0, 6.5, 7.0, and 7.5. Spontaneous deamidation of the gliadin peptide was excluded (data not shown). Fluorescence labelled peptide *α2(56–68) was deamidated and cross linked by tTG. Increasing pH values

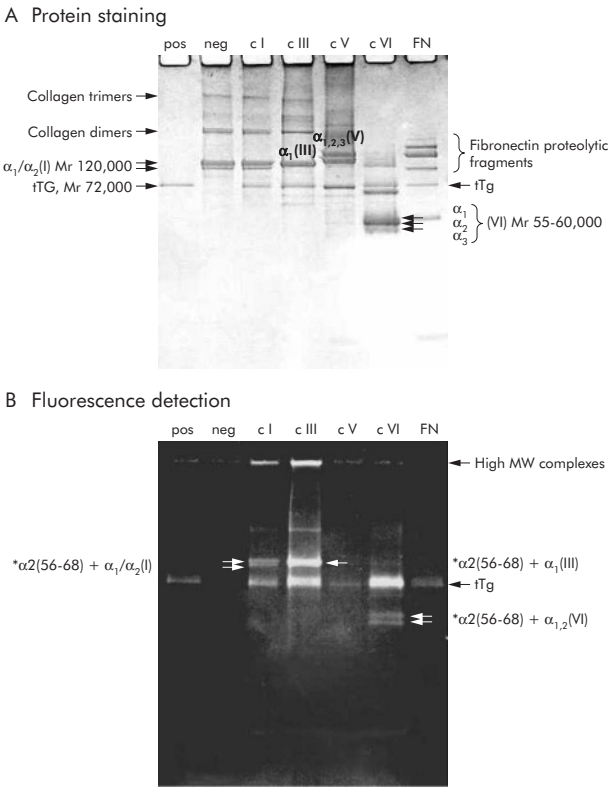

Figure 3 Tissue transglutaminase (tTG) mediated binding of gliadin peptide *α2(56–68) to extracellular matrix molecules. (A) Protein staining (Coomassie blue) for localisation of tTG and collagen types I, III, V, and VI, and fibronectin (FN), after reducing 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. (B) Simultaneous fluorescence detection of peptide *α2(56–68) incorporated into the same panel of extracellular matrix proteins. Only α-chains of collagen types I, III, and VI (molecular weight (Mr) 120 000 and 60 000, respectively) and tTG itself (Mr 72,000) served as gliadin acceptor substrates. No incorporation of the gliadin peptide *α2(56–68) was noted into collagen type V or proteolytic fragments of fibronectin. The positive control shows incorporation of the gliadin peptide into tTG. The negative control without tTG demonstrates that there is no spontaneous binding of gliadin to collagen type I in the absence of tTG.

caused a continuous increase in incorporation of the peptide into tTG which was paralleled by the creation of high molecular weight complexes formed by tTG-gliadin multimers (fig 2). As cross linking also occurred at pH values as low as 6.0, tTG-gliadin complexes should also be formed in inflamed intestine.

tTG catalysed cross linking of gliadin peptides with extracellular matrix molecules

Fluorescence labelled peptide *α2(56–68) was used to investigate tTG mediated cross linking of gliadins to ECM proteins. Both α-chains of collagen type I (with the $\alpha_1(I)_2$ α_2(I) heterotrimer) and the α_1-chain of collagen type III (with the $\alpha_1(III)_3$ homotrimer) were cross linked with the gliadin peptide. Incorporation of the gliadin peptide into collagen type VI (with the α_1(VI), α_2(VI), α_3(VI) heterotrimer) seemed to be restricted to the $\alpha_2 > \alpha_1$ chains. Although α_1(VI) was hardly detectable with the protein stain, a fluorescence signal was clearly assigned to this chain. Native collagen V (with the α_1(V), α_2(V), α_3(V) heterotrimer) and fibronectin, as well as native collagen type IV and laminin (not shown), showed no incorporation of the gliadin peptide (fig 3).

tTG catalysed colocalisation of gliadin peptides with extracellular matrix molecules in tissues

Primate oesophagus showed a distinct distribution pattern of fibronectin and collagens. Whereas fibronectin was found in the lamina muscularis mucosae (fig 4A), collagen types I and III displayed an overlapping staining pattern and were mainly localised in the lamina propria mucosae and in only some fibres of the lamina muscularis mucosae (fig 4B, coll(I)). Collagen VI showed a broader distribution with strong reactivity of the lamina propria mucosae and the lamina muscularis mucosae (fig 4C). Immunostaining for tTG (fig 4E) was superimposable on the pattern obtained after incubation of the sections with the *α2(56–68) gliadin peptide, yielding the characteristic honeycomb endomysial pattern with main reactivity in the lamina muscularis mucosae (fig 4D). Colocalisation of the gliadin peptide with tTG, and the finding that staining with the gliadin peptide was calcium dependent and absent in EDTA containing buffer (data not shown), clearly demonstrates that extracellular tTG can bind gliadin to the matrix. The almost exclusive staining of the lamina muscularis mucosae for tTG (fig 4E) and fibronectin (fig 4A) and not for the major interstitial collagen types I and III (fig 4B), is in line with the reported non-covalent association of tTG with these fibronectin fibres.[40–42]

In contrast, preincubation of the tissue sections with additional tTG showed a completely different pattern with strong staining of the lamina propria mucosae, thus indicating that an excess of tTG, as is secreted in the coeliac mucosae, will favour binding of tTG to regions with high content of collagens (fig 4F). Accordingly, concurrent incubation of the sections with the *α2(56–68) gliadin peptide and increasing amounts of human recombinant tTG (0.5–2.0 μg/section; fig 4G and H) yielded a diffuse staining pattern with emerging fluorescence in the lamina propria mucosae. This was caused by the gliadin peptide being linked to collagens by extrinsic tTG, in addition to the lamina muscularis mucosae, due to the gliadin peptide being linked to intrinsic tTG by autocatalysis.

IgA autoantibodies to collagens

An ELISA was performed to measure serum IgA autoantibodies to collagen types I, III, V, and VI, and fibronectin in patients with active coeliac disease and healthy controls.

Compared with controls, coeliac patients demonstrated significantly elevated titres of IgA autoantibodies to collagen types I, III, V, and VI ($p<0.05$, Mann Whitney U test) (fig 5). Patients on a gluten free diet showed IgA titres not distinguishable from the titres of healthy controls (data not shown). Notably, IgG autoantibody titres to these collagens did not differ between coeliacs compared with controls. No differences in titres of IgA or IgG autoantibodies to fibronectin were found in coeliac patients or controls.

DISCUSSION

Despite several data suggesting the involvement of all gliadin fractions in the pathogenesis of coeliac disease, experimental studies have been restricted to a defined set of immunodominant gliadin and glutenin peptides.[10–17] Site directed deamidation of gliadin peptides by tTG depends on the primary structure of the peptides and some consensus sequences have been identified. Whereas peptides containing the sequence motifs QXP or QXX followed by the hydrophobic residues F, Y, M, W, L, I, or V (with X for any amino acid) are preferred substrates for tTG, the sequences QP or QXXP are not substrates of the enzyme.[21] tTG specifically deamidates glutamine 65 in the immunodominant gliadin peptide α2(56–68) that contains the core sequence PQPQLPY generating PQPELPY.[39] Here we used an alternative approach

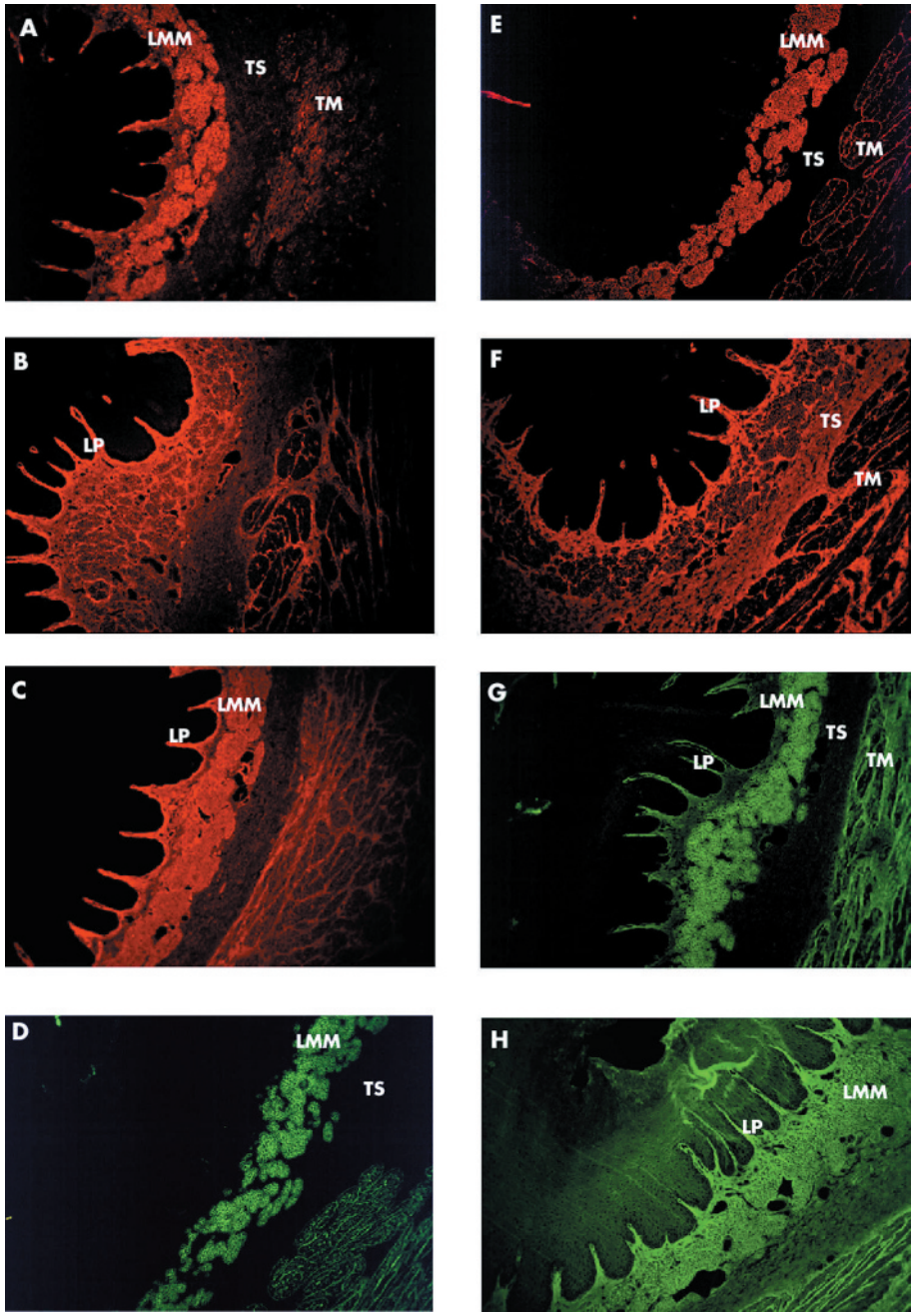

Figure 4 Localisation of extracellular matrix proteins and gliadin in oesophageal sections. Indirect immunofluorescence staining of monkey oesophagus (magnification 25×) with tetramethyl-rhodamine (TRITC) labelled polyclonal antibodies against (A) fibronectin, mainly staining the lamina muscularis mucosae, (B) collagen type I, mainly localised in the lamina propria mucosae and yielding an identical staining pattern to collagen type III (not shown), and (C) collagen type VI, being prominent both in the lamina propria mucosae and the lamina muscularis mucosae. Incubation of the sections with the fluorescence labelled gliadin peptide *α2(56–68) (D) or with a TRITC labelled monoclonal antibody against tissue transglutaminase (tTG) (E) demonstrates the honeycomb staining pattern of the lamina muscularis mucosa, characteristic of endomysial autoantibodies in coeliac disease. Detection of tTG after preincubation with additional tTG yielded a distribution pattern resembling staining for collagen types I and III (F). (G, H) Addition of *α2(56–68) together with tTG (0.5 or 2.0 μg/section for (F) and (G), respectively) produced a diffuse staining pattern encompassing the lamina muscularis mucosae and the lamina propria mucosae. LMM, lamina muscularis mucosae; LP, lamina propria; TM, tunica muscularis; TS, tela submucosae.

to verify this consensus sequence in the gliadin peptides α2(56–68) and α2(58–88) as the only recognition site for tTG.

Furthermore, the fluorescence labelled gliadin peptide *α2(56–68) was used to show that tTG promotes the autocatalytic incorporation of this peptide into tTG itself. This again occurs via the substrate glutamine residue at position 65, resulting in the formation of high molecular weight complexes containing autocatalytic tTG multimers, and tTG-gliadin peptide cross links. We observed this incorporation and complex formation at lower pH values also, conditions which were formerly described to favour deamidation over cross linking.[13] Therefore, an environment with a pH as low as pH 6 which can prevail in inflammation still leads to tTG catalysed gliadin cross linking. Recently, using matrix assisted laser desorption ionisation and mass spectrometry of enzymatically digested tTG-gliadin complexes, six lysine residues of tTG were identified as cross linking sites with gliadin peptides.[43] It is still not clear whether tTG-gliadin cross links are directly involved in the pathogenesis of coeliac disease. However, the presence of serum IgA and IgG antibodies directed against these cross links (own unpublished results) in addition to antibodies against gliadin, tTG, or deamidated gliadins, supports a possible role of these complexes in the immunopathogenesis of coeliac disease.

An important causative factor for the development of the small intestinal coeliac lesion is the availability of undegraded toxic gliadin sequences in the intestinal mucosa. In this vein, identification of the highly immunogenic 33-mer peptide of α2-gliadin (56–88) which is fairly resistant to digestion by intestinal brush border enzymes and likely reaches the lamina propria where T cell activation occurs, was an important finding.[12 44] The in vivo toxicity of the core sequence (62–75) of this 33-mer peptide was proven in a

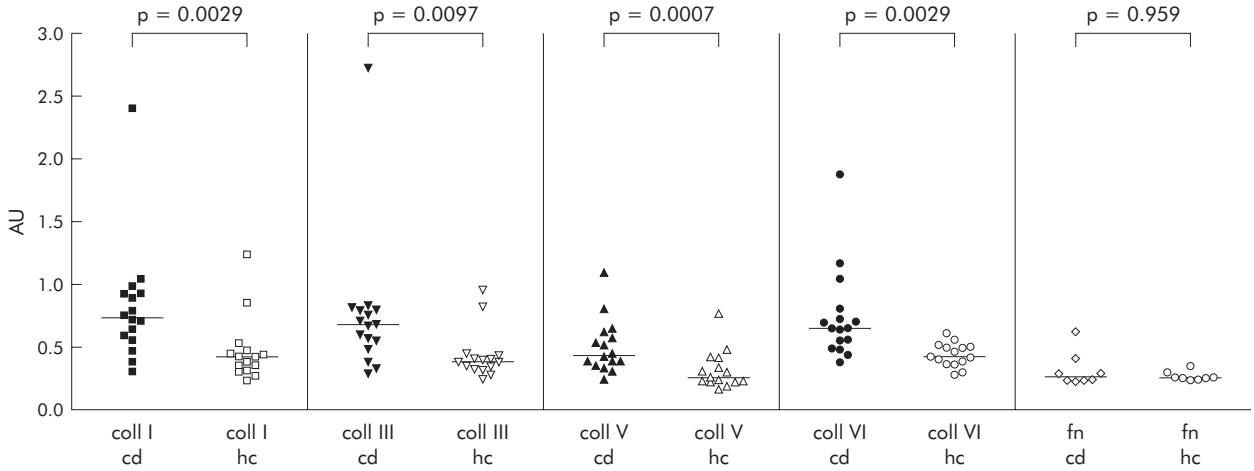

Figure 5 IgA autoantibodies against collagen (coll) types I, III, V, and VI, and fibronectin (fn). Titres of IgA autoantibodies to collagen types I, III, V, and VI, and fibronectin of coeliac patients (cd) and non-coeliac healthy controls (hc), as measured by enzyme linked immunosorbent assay, are shown in a scatterplot. The corresponding medians are marked. IgA (and IgG) autoantibody titres to fibronectin did not differ between coeliacs and controls. Each measurement was done in duplicate.

recent report.[45] Furthermore, an immunohistochemical study demonstrated that gliadin colocalised with tTG in the duodenal mucosa of untreated coeliac patients and controls. This colocalisation was mainly found in the epithelial and subepithelial areas in active coeliac disease but was restricted to the lamina propria in controls.[46] However, it remains unclear how far an altered expression and distribution pattern of tTG,[46] or rather the known increased tTG activity in the duodenal mucosa of patients with coeliac disease, contributes to coeliac disease pathogenesis.[15 47 48]

Our demonstration of tTG mediated gliadin incorporation into collagen types I, III, and VI, which are predominant interstitial matrix components in the lamina propria, supports the concept that due to immobilisation in the ECM of coeliac patients, local availability of gliadins can reach increased concentrations in vivo. This may be potentiated in coeliac disease which is characterised by an increased volume and matrix content of the lamina propria.[49] We observed no cross linking of the gliadin peptide to collagen type V, thus indicating that this collagen does not serve as a gliadin acceptor substrate. None the less, the telopeptide of collagen type V was described as a glutamine donor substrate for tTG,[50] therefore allowing complex formation with tTG itself. In addition, no incorporation of the gliadin peptide was found in the basement membrane constituents collagen type IV, laminin, and in fibronectin matrix components, the patterns of which were reported to be disorganised in coeliac disease.[49]

Intrinsic tTG colocalises non-covalently with fibronectin in the lamina muscularis mucosae.[40–42] However, our data proved that excess tTG, as secreted in coeliac disease, will change the binding behaviour and favour association of tTG and tTG-gliadin complexes with collagens, especially in the lamina propria. The resulting increased tTG activity followed by an increase in tTG modified gliadins, gliadin-collagen, or gliadin-tTG complexes in the coeliac lamina propria could favour progression and chronicity of coeliac disease. Haptenisation of immunogenic gliadin peptides by collagens I, III, and VI as well as by tTG itself, and vice versa, likely enhances the immune reaction to these gliadin peptides, to tTG, and to collagens. While the occurrence of IgA autoantibodies to tTG is pathognomonic for coeliac disease,[18] our finding of significantly increased IgA autoantibodies against collagen types I, III, V, and VI in patients with active coeliac disease compared with non-diseased controls is of particular interest. It is assumed that epitope spreading from gliadins to tTG due to hapten-like gliadin-tTG complexes occurs.[15] Accordingly, expansion of the mucosal humoral response from gliadins/deamidated gliadins to cross links of gliadin with collagens (types I, III, and VI), or supposedly of tTG with collagen type V, is likely. This haptenisation of collagens by gliadins combined with a switch from IgA to IgG class antibodies could be responsible for the increased association of coeliac disease with connective tissue diseases.[51] In this context, it is of interest to follow up our patients with increased IgA autoantibodies against collagens for development of connective tissue diseases.

In summary, the confirmation of defined tTG-recognition sites in gliadin peptides in combination with demonstration of cross linking of these preferred peptides to tTG and certain ECM proteins confirms the essential role of tTG in creating immunogenic neoepitopes and enhancing antigenic presentation of gliadins. We demonstrated that tTG catalysed modifications are not restricted to single gliadins or gliadin epitopes as a wide panel of gliadins can be modified by tTG. This finding verifies prior reports that applies search algorithms to identify preferred tTG substrates and that yielded matches in the gluten (wheat), hordein (rye), and secalin (barley) databases.[21] Therefore, the intestinal immune response of coeliac patients is even more complex due to enhanced tTG mediated binding of gliadins or related immunogenic peptides to matrix components, which in addition creates long term reservoirs of antigenically potentiated gluten components. Furthermore, a mucosal immune reaction against extracellular matrix proteins, as mirrored by IgA autoantibodies to these proteins, may play a role in secondary autoimmune diseases that are associated with coeliac disease.

ACKNOWLEDGEMENTS

We thank Dr Chaitan Khosla and Dr Justin Piper for the kind gift of the recombinant tTG plasmid and Maria Leidenberger and Ingrid Zöller-Utz for excellent technical support.

Authors' affiliations
W Dieterich, B Esslinger, D Trapp, E Hahn, Department of Medicine I, University of Erlangen-Nuernberg, Germany
T Huff, Department of Biochemistry, University of Erlangen-Nuernberg, Germany
W Seilmeier, H Wieser, German Research Centre of Food Chemistry, Garching, Germany

D Schuppan, Department of Medicine I, University of Erlangen-Nuernberg, Germany, and Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA

This study was supported by grants Schu 646/11-3 from the Deutsche Forschungsgesellschaft and by grants of the commission of the European Communities, Coeliac EU-cluster program ''Quality of Life and Management of Living Resources, QLRT-1999-00037''.

Conflict of interest: None declared.

REFERENCES

1 **Van de Kamer JH**, Weijers HA, Dicke WK. Coeliac disease IV: An investigation into the injurious constituents of wheat in connection with coeliac disease. *Acta Paediatr* 1953;**42**:223–31.
2 **Marsh MN**. Gluten, major histocompatibility complex, and the small intestine: A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('coeliac sprue'). *Gastroenterology* 1992;**102**:330–54.
3 **Wieser H**. Chemistry of gliadins. *Eur J Gastroenterol Hepatol* 1991;**3**:102–7.
4 **Seilmeier W**, Wieser H. Comparative investigations of gluten proteins from different wheat species. IV. Reactivity of gliadin fractions and components from different wheat species in a commercial immunoassay. *Eur Food Res Technol* 2003;**217**:360–4.
5 **Sturgess R**, Day P, Ellis HJ, *et al*. Wheat peptide challenge in coeliac disease. *Lancet* 1994;**343**:758–61.
6 **Mantzaris G**, Jewell DP. In vivo toxicity of a synthetic dodecapeptide from A gliadin in patients with coeliac disease. *Scand J Gastroenterol* 1991;**26**:392–8.
7 **De Ritis G**, Auricchio S, Jones HW, *et al*. In vitro (organ culture) studies of the toxicity of specific A-gliadin peptides in coeliac disease. *Gastroenterology* 1988;**94**:41–9.
8 **Carroccio A**, Iacona G, D'Amico D, *et al*. Production of anti-endomysial antibodies in cultured duodenal mucosa: usefulness in coeliac disease diagnosis. *Scand J Gastroenterol* 2002;**37**:32–8.
9 **Cornell H**, Wieser H, Belitz HD. Characterization of the gliadin-derived peptides which are biologically active in coeliac disease. *Clin Chim Acta* 1992;**213**:37–50.
10 **Arentz-Hansen H**, Körner R, Molberg Ø, *et al*. The intestinal T cell response to α-gliadin in adult coeliac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J Exp Med* 2000;**191**:603–12.
11 **Anderson RP**, Degano P, Godkin AJ, *et al*. In vivo antigen challenge in coeliac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med* 2000;**6**:337–342.
12 **Shan L**, Molberg Ø, Parrot I, *et al*. Structural basis for gluten intolerance in coeliac sprue. *Science* 2002;**297**:2275–9.
13 **Fleckenstein B**, Molberg Ø, Qiao SW, *et al*. Gliadin T cell epitope selection by tissue transglutaminase in coeliac disease. *J Biol Chem* 2002;**277**:34109–16.
14 **Arentz-Hansen H**, McAdam SN, Molberg Ø, *et al*. Coeliac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology* 2002;**123**:803–9.
15 **Molberg Ø**, McAdam SN, Koerner R, *et al*. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in coeliac disease. *Nat Med* 1998;**4**:713–17.
16 **Van de Wal Y**, Kooy Y, van Veelen P, *et al*. Selective deamidation by tissue transglutaminase strongly enhances gliading-specific T cell reactivity. *J Immunol* 1998;**161**:1585–8.
17 **Ellis HJ**, Pollock EL, Engel W, *et al*. Investigation of the putative immunodominant T cell epitopes in coeliac disease. *Gut* 2003;**52**:212–17.
18 **Dieterich W**, Ehnis T, Bauer M, *et al*. Identification of tissue transglutaminase as the autoantigen of coeliac disease. *Nat Med* 1997;**3**:797–801.
19 **Griffin M**, Casadio R, Bergamini CM. Transglutaminases: nature's biological glues. *Biochem J* 2002;**368**:377–96.
20 **Larre C**, Chiarello M, Blanloeil Y, *et al*. Gliadin modifications catalyzed by guinea pig liver transglutaminase. *J Food Biochem* 1993;**17**:267–82.
21 **Vader WL**, de Ru A, van der Wal Y, *et al*. Specificity of tissue transglutaminase explains cereal toxicity in coeliac disease. *J Exp Med* 2002;**195**:643–9.
22 **Osman AA**, Günnel T, Dietl A, *et al*. B cell epitopes of gliadin. *Clin Exp Immunol* 2000;**121**:248–54.
23 **Aleanzi M**, Demonte AM, Esper C, *et al*. Coeliac disease: antibody recognition against native and selectively deamidated gliadin peptides. *Clin Chem* 2001;**47**:2023–8.
24 **Chirdo FG**, Rumbo M, Carabajal P, *et al*. Determination of anti-ω-gliadin antibodies in serologic tests for coeliac disease. *Scand J Gastroenterol* 2000;**35**:508–16.
25 **Ensari A**, Marsh MN, Moriarty KJ, *et al*. Studies in vivo of ω-gliadins in gluten sensitivity (coeliac sprue disease). *Clin Sci* 1998;**95**:419–24.
26 **Ciclitira PJ**, Evans DJ, Fagg NLK, *et al*. Clinical testing of gliadin fractions in coeliac patients. *Clin Sci* 1984;**66**:357–64.
27 **Van de Wal Y**, Kooy YMC, van Veelen P, *et al*. Glutenin is involved in the gluten-driven mucosal T cell response. *Eur J Immunol* 1999;**29**:3133–9.
28 **Vader W**, Kooy Y, van Veelen P, *et al*. The gluten response in children with coeliac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology* 2002;**122**:1729–37.
29 **Molberg O**, Solheim Flaete N, Jensen T, *et al*. Intestinal T-cell responses to high-molecular-weight glutenins in coeliac disease. *Gastroenterology* 2003;**125**:337–44.
30 **Martucci S**, Corazza GR. Spreading and focusing of gluten epitopes in coeliac disease. *Gastroenterology* 2002;**122**:2072–5.
31 **Mothes T**, Osmann AA, Seilmeier W, *et al*. The activity of single gliadin components in a foetal chick intestine assay for coeliac disease. *Eur Food Res Technol* 1999;**210**:93–6.
32 **Seilmeier W**, Valdez I, Mendez E, *et al*. Comparative investigations of gluten proteins from different wheat species. II. Characterization of ω-gliadins. *Eur Food Res Technol* 2001;**212**:355–63.
33 **Wieser H**, Engel W. Synthesis and purification of peptides used for studies on coelic disease. In: Stern M, eds. *Proceedings of the 17th Meeting on the Working Group on Prolamin Analysis and Toxicity*. Zwickau: Verlag Wissenschaftliche Scripten, 2003:121–5.
34 **Lorand L**, Lockridge OM, Campbell LK, *et al*. Transamidating enzymes. II. A continuous fluorescent method suited for automating measurements of factor XIII in plasma. *Anal Biochem* 1971;**44**:221–31.
35 **Dieterich W**, Trapp D, Esslinger B, *et al*. Autoantibodies of patients with coeliac disease are insufficient to block tissue transglutaminase activity. *Gut* 2003;**52**:1562–6.
36 **Schuppan D**, Schmid M, Somasundaram R, *et al*. Collagens in the liver extracellular matrix bind hepatocyte growth factor. *Gastroenterology* 1998;**114**:139–52.
37 **Rühl M**, Somasundaram R, Schönfelder I, *et al*. The epithelial mitogen keratinocyte growth factor binds to collagens via the consensus sequence glycine-proline-hydroxyproline. *J Biol Chem* 2002;**277**:26872–8.
38 **Somasundaram R**, Rühl M, Schäfer B, *et al*. Interstitial collagens I, III, and VI sequester and modulate the multifunctional cytokine oncostatin M. *J Biol Chem* 2002;**277**:3242–6.
39 **Piper JL**, Gray GM, Khosla Ch. High selectivity of human tissue transglutaminase for immunoactive gliadin peptides: Implications for coeliac sprue. *Biochemistry* 2002;**41**:386–93.
40 **LeMosy EK**, Erickson HP, Beyer WF, *et al*. Visualization of purified fibronectin-transglutaminase complexes. *J Biol Chem* 1992;**11**:7880–5.
41 **Gaudry CA**, Verderio E, Aeschlimann D, *et al*. Cell surface localization of tissue transglutaminase is dependent on a fibronectin-binding site in its N-terminal β-sandwich domain. *J Biol Chem* 1999;**274**:30707–14.
42 **Akimov SS**, Belkin AM. Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: a role in TGFβ-dependent matrix deposition. *J Cell Sci* 2001;**114**:2989–3000.
43 **Fleckenstein B**, Qiao SW, Larsen MR, *et al*. Molecular characterization of covalent complexes between tissue transglutaminase and gliadin peptides. *J Biol Chem* 2004;**279**:17607–16.
44 **Matysiak-Budnik T**, Candalh C, Dugave C, *et al*. Alterations of the intestinal transport and processing of gliadin peptides in coeliac disease. *Gastroenterology* 2003;**125**:696–707.
45 **Fraser JS**, Engel W, Ellis HJ, *et al*. Coeliac disease: in vivo toxicity of the putative immunodominant epitope. *Gut* 2003;**52**:1698–702.
46 **Ciccocioppo R**, Di Sabatino A, Ara C, *et al*. Gliadin and tissue transglutaminase complexes in normal and coeliac duodenal mucosa. *Clin Exp Immunol* 2003;**134**:516–24.
47 **Brusco G**, Muzi P, Ciccocioppo R, *et al*. Transglutaminase and coeliac disease: endomysial reactivity and small bowel expression. *Clin Exp Immunol* 1999;**118**:371–5.
48 **Esposito C**, Paparo F, Caputo I, *et al*. Expression and enzymatic activity of small intestinal tissue transglutaminase in coeliac disease. *Am J Gastroenterol* 2003;**98**:1813–20.
49 **Verbeke S**, Gotteland M, Fernandez M, *et al*. Basement membrane and connective tissue proteins in intestinal mucosa of patients with coeliac disease. *J Clin Pathol* 2002;**55**:440–5.
50 **Kleman JP**, Aeschlimann D, Paulsson M, *et al*. Transglutaminase-catalyzed cross-linking of fibrils of collagen V/XI in A204 rhabdomyosarcoma cells. *Biochemistry* 1995;**34**:13768–75.
51 **Ventura A**, Magazzu G, Greco L. Duration of exposure to gluten and risk for autoimmune disorders in patients with coeliac disease. SIGEP Study Group for Autoimmune Disorders in Coeliac Disease. *Gastroenterology* 1999;**117**:297–303.